Structures of *Leishmania major* Pteridine Reductase Complexes Reveal the Active Site Features Important for Ligand Binding and to Guide Inhibitor Design

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Pteridine reductase (PTR1) is an NADPH-dependent short-chain reductase found in parasitic trypanosomatid protozoans. The enzyme participates in the salvage of pterins and represents a target for the development of improved therapies for infections caused by these parasites. A series of crystallographic analyses of *Leishmania major* PTR1 are reported. Structures of the enzyme in a binary complex with the cofactor NADPH, and ternary complexes with cofactor and biopterin, 5,6-dihydrobiopterin, and 5,6,7,8-tetrahydrobiopterin reveal that PTR1 does not undergo any major conformational changes to accomplish binding and processing of substrates, and confirm that these molecules bind in a single orientation at the catalytic center suitable for two distinct reductions. Ternary complexes with cofactor and CB3717 and trimethoprim (TOP), potent inhibitors of thymidylate synthase and dihydrofolate reductase, respectively, have been characterized. The structure with CB3717 reveals that the quinazoline moiety binds in similar fashion to the pterin substrates/products and dominates interactions with the enzyme. In the complex with TOP, steric restrictions enforced on the trimethoxyphenyl substituent prevent the 2,4-diaminopyrimidine moiety from adopting the pterin mode of binding observed in dihydrofolate reductase, and explain the inhibition properties of a range of pyrimidine derivatives. The molecular detail provided by these complex structures identifies the important interactions necessary to assist the structure-based development of novel enzyme inhibitors of potential therapeutic value.

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**Introduction**

Infection by trypanosomatid protozoans, e.g. *Trypanosoma* and *Leishmania* species, causes a range of serious human diseases.¹ Current treatments of these infections involve drugs such as sodium stibogluconate and nifurtimox, which induce serious side-effects and this, together with an increase in drug-resistant parasites, has created an urgent requirement for new and more effective treatments.² The ideal enzyme targets for the development of such new antimicrobial drugs are those that are essential for the survival of the parasite, and either absent from the human host or display markedly differing substrate specificities. Our improved knowledge of trypanosomatid
biology and biochemistry, greatly facilitated by developments in molecular genetics and genome sequencing efforts, promotes understanding of how existing drugs function and is helping to identify potential targets for chemotherapeutic attack.\textsuperscript{3,4} Whilst there are increased opportunities to target recently discovered enzymes there are also well-studied systems that still have much to offer. Our present study concerns the \textit{Leishmania} pteridine reductase (PTR1; EC 1.5.1.33), which is involved in folate/pterin metabolism and, as will be explained, presents a link to the well-known therapeutic targets dihydrofolate reductase (DHFR; EC 1.5.1.3) and thymidylate synthase (TS; EC 2.1.1.45).

Folate metabolism, in particular DHFR and TS, have been targeted successfully for the treatment of cancer and microbial infection.\textsuperscript{5-10} TS catalyses the conversion of \textit{dUMP} to \textit{dTMP} using the cofactor N\textsubscript{5}, N\textsubscript{10}-methylene tetrahydrofolate (THF) as both the C-donor and reductant, whilst DHFR maintains the THF pool by the NADPH-dependent reduction of dihydrofolate (DHF). Inhibition of either enzyme limits the supply of \textit{dTMP} required for DNA synthesis, thus curtailing replication and leading to cell death. In most organisms, DHFR and TS are separate entities. \textit{Trypanosomatid} protozoans possess a bifunctional DHFR-TS, which has been structurally characterised.\textsuperscript{11}

Since folates and pterins are essential for \textit{trypanosomatid} growth, antifolates targeting DHFR, in principle, should provide an ideal treatment. However, DHFR inhibitors are largely ineffective for the control of \textit{trypanosomatid} infections, partly due to the presence of PTR1.\textsuperscript{12} This short-chain dehydrogenase/reductase (SDR) family member exhibits a broad NADPH-dependent pteridine reductase activity, capable of reducing unconjugated (biopterin) and conjugated (folate) pterins from either the oxidized or dihydro-state (Figure 1). This activity is essential for parasite growth \textit{in vitro}.\textsuperscript{12} The biochemical activities of PTR1 overlap those of DHFR but PTR1 is less susceptible to inhibition by classical antifolates such as methotrexate (MTX); thus, it can function as a metabolic by-pass to alleviate DHFR inhibition.\textsuperscript{13} However, an inhibitor of PTR1 has the potential to act in concert with known antifolates to provide a novel approach to the treatment of \textit{trypanosomatid} infection.\textsuperscript{14}

The kinetics and stereochemical course of the reductions catalyzed by PTR1 have been studied together with analysis of a library of inhibitors.\textsuperscript{14,15} Crystal structures are available of the enzyme from \textit{Leishmania major} (\textit{Lm}\textsubscript{PTR1}) in ternary complexes with cofactor and 7,8-dihydrobiopterin (DHB),\textsuperscript{16} the archetypal antifolate drug MTX (4-amino-N10-methyl-pteroylglutamic acid; Figure 2)\textsuperscript{16} and 2,4,6-triaminoquinazoline (TAQ).\textsuperscript{17} Other laboratories have reported the 2.7 A˚ resolution crystal structure of PTR1 from \textit{Leishmania tarentolae}\textsuperscript{18} and the crystallization of the enzyme from \textit{Trypanosoma cruzi}.\textsuperscript{19}

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure1.png}
\caption{The two-stage reduction of biopterin (2-amino-6-(1,2-dihydroxypropyl)pteridin-4(3H)-one) to 7,8-dihydrobiopterin (2-amino-7,8-dihydro-6-(1,2-dihydroxypropyl)pteridin-4(3H)-one), and then to 5,6,7,8-tetrahydrobiopterin (2-amino-5,6,7,8-tetrahydro-6-(1,2-dihydroxypropyl)pteridin-4(3H)-one) catalyzed by PTR1. Each stage requires one reducing equivalent provided by the cofactor NADPH. The carbon atoms (C6 and C7) that accept hydride from the cofactor are marked with an asterisk (*).}
\end{figure}

We set out to characterize PTR1–ligand complexes to derive important molecular details necessary to understand the enzyme and to underpin a structure-based approach to inhibitor development. Structural analyses of discrete steps along the two-stage PTR1 catalytic process (Figure 1) are reported. The binary complex with cofactor (PTR1:NADPH) corresponds to the state prior to binding substrate. The ternary complexes with biopterin, and DHB represent the initial substrate complexes for stages I and II, respectively. The DHB complex also represents the product complex at the end of stage I, and finally the 5,6,7,8-tetrahydrobiopterin (THB) complex represents the product complex at the end of stage II.

The potent TS inhibitor CB3717 (N10-propargyl-5,8-dideazafolic acid, Figure 2), resembles DHF, though with the addition of a propargyl group, and we reasoned that it might bind to PTR1, thereby...
raising the potential to design a molecule capable of inhibiting both PTR1 and TS. Structures of CB3717 in complex with TS from Escherichia coli,20 Lactobacillus casei,21 Pneumocystis carinii TS22 and the bifunctional DHFR-TS of L. major11 are available for comparative purposes.

One of the most successful antimicrobial antifolates is trimethoprim (5-((3,4,5-trimethoxyphenyl)methyl)-2,4-diaminopyrimidine, TOP; Figure 2).9 TOP is a potent inhibitor of DHFR23 and structures with the enzyme from Mycobacterium tuberculosis,24 Staphylococcus aureus,25 E. coli and chicken26 are known. A structure of PTR1 in complex with TOP was sought to provide a different molecular framework in the enzyme active site, one that is necessary to understand PTR1 inhibition by a series of 2,4-diaminopyrimidine derivatives.

Results and Discussion

Crystallographic details

Five medium-resolution crystal structures of LmPTR1 ligand complexes have been determined with diffraction data recorded using synchrotron radiation. The structures include the binary complex with cofactor NADPH (2.65 Å resolution), ternary complexes of oxidized cofactor and biopterin (2.40 Å) and THB (2.55 Å) and two ternary complexes with the inhibitors CB3717 (2.70 Å) and TOP (2.60 Å). The structures all display space group $P_{21}2_12_1$ and are isomorphous. A homotetramer, of molecular mass approximately 120 kDa, constitutes the asymmetric unit and gives a Matthews coefficient, $V_M$, of 2.8 Å³ Da⁻¹ and 55% (v/v) solvent volume. The PTR1 tetramer displays 222 point group symmetry. Most of the protein residues are well defined by the electron density, with only two segments disordered in all subunits; these are the surface loops comprising residues 75–80 and 120–130. In addition, residues 230–240 are disordered in two subunits. This is discussed later. In the structures, the cofactor, biopterin, THB and pteridine component of CB3717 are well ordered in each of the four active sites in the asymmetric unit. The remainder of CB3717 is poorly ordered. The inhibitor TOP is observed in two active sites only. Further information is given in Table 1.

Overall structure and cofactor binding

PTR1 is a member of the SDR super family of enzymes, of which there are over 3000 members with sequence identities at the 15–30% level.27,28 The subunit displays the extended double-Rossmann fold typical of the SDR family, which is based on a central seven-stranded parallel $\beta$-sheet with three $\alpha$-helices on either side (Figure 3(a)). In addition, PTR1 has a short helix $\alpha_6$ following $\beta_6$, and two short strands between $\beta_3$ and $\alpha_3$ framing a disordered loop (residues 75–80). Another, frequently disordered section of the PTR1 structure occurs between $\beta_4$ and $\alpha_4$ (residues 120–130). Residues 230–240 form the so-called substrate-binding loop29 between $\beta_6$ and $\alpha_6$, a common structural feature of the SDR family. Generally, this loop is disordered or in an open conformation in crystal structures of the apo form or binary complexes of family members. Once substrate or inhibitors bind, the loop adopts an ordered and closed conformation. In the present structures, this substrate-binding loop is ordered in two of the four subunits that constitute the asymmetric unit, due to interactions within the crystal lattice, in particular the associations of Met233 and Pro234 with Tyr114 from a symmetry mate (not shown).

A comparison of seven LmPTR1 structures, the five reported here plus the MTX and DHB complexes,16 by a least-squares fit of all superimposable $C^\alpha$ atoms in the functional tetramers
(approximately 1035 atoms for each structure), gives a root-mean-square deviation (r.m.s.d.) range of 0.17–0.58 Å with a mean of 0.34 Å. The smallest deviation is observed for the overlay of the ternary DHB and THB complexes, and the largest is observed between the binary cofactor complex paired with the ternary MTX complex. Since the structures were refined independently, this indicates a high level of structural conservation of the protein, irrespective of whether PTR1 is in a binary or a ternary complex, or whether substrate, product or an inhibitor is bound. Inspection of the superimposed molecules (not shown) confirms this high degree of structural conservation, which extends to the positions of side-chains, the conformation of the cofactor, and even the positions of numerous water molecules. We also note structural conservation within the asymmetric unit of each complex. Such consistency indicates that PTR1 does not undergo large conformational changes upon binding and processing substrate, and that it is necessary to describe only one enzyme active site, in this case chosen arbitrarily as that formed mostly by subunit A.

The PTR1 active site is an elongated L-shaped cleft (Figure 3(b)) approximately 22 Å × 15 Å, created mainly by C-terminal sections of strands β1 to β6, and sections contributed from α1, α4 and α5 together with the residues on the loop between β6 and α6. The C terminus of a partner subunit blocks one end of the active site, with Arg287 (the prime (') identifies a contribution from another subunit) directed towards the catalytic center and near Asp181. The cofactor binds in the active site in an extended conformation, with the nicotinamide creating the floor of the catalytic center; Phe113 forms an overhang under which the pterin-binding pocket is formed. The adenine residue adopts an anti conformation and the nicotinamide moiety adopts a syn conformation with respect to their ribose groups. In the binary complex, the pterin-binding site is occupied by water molecules with a molecule of ethylene glycol binding to Asp181 and Arg287 (Figure 4). The MTX complex also shows ethylene glycol binding in this region of the active site.

A complex network of hydrogen bonds organize the enzyme active site and position the cofactor (not shown).16,17 Notably, Asn147 forms interactions with Lys198 and Ser111, positioning the latter two residues to bind the nicotinamide ribose. The amide group of Ser111 is also able to donate a hydrogen bond to O4 of the adenine ribose. Arg17 interacts with the main-chain carbonyl group of Val228 and plays a critical role in binding the cofactor pyrophosphate. The main-chain amide and

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<th>PTR1:NADPH+ THB</th>
<th>PTR1:NADPH: CB3717</th>
<th>PTR1:NADPH: TOP</th>
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Values in parentheses pertain to the highest resolution shell (width = 0.1 Å). DPI = diffraction-component precision index.52
carbonyl groups of Ser227 form hydrogen bonds with the carboxamide group of the nicotinamide, which in turn interacts with the nearby cofactor phosphate group. The C2, C4, and C6 groups of the nicotinamide participate in three C–H...O hydrogen bonds, interacting with a phosphodiester O of the cofactor, and the carbonyl oxygen atoms of Gly225 and Val180, respectively (not shown). Though weak, these interactions assist the association of protein with cofactor and help to align the nicotinamide to facilitate hydride transfer from C4. These C–H...O associations are commonly observed in SDR structures.

**Substrate and product complexes**

The ternary complex structures with biopterin, DHB and THB all have the pterin ligands bound in the same orientation and participating in virtually identical interactions with the enzyme and cofactor (Figures 4 and 5) as described for the DHB complex. An interesting observation from early studies on DHFR is that the pteridine moiety of MTX binds in the active site in an orientation different from that adopted by the substrate DHF. Likewise, in PTR1, the substrates bind with the pteridine rotated about the N2–N5 axis by 180° relative to MTX.

Average surface-accessible areas have been determined using a solvent probe of radius 1.4 Å.
**Figure 5.** A representation of biotin binding to the *Ln*PTR1 active site. Broken lines represent potential hydrogen bonds and the blocks of color are the same as in Figure 3.

**Figure 6.** CB3717 binding to *Ln*PTR1. (a) Stereoview showing the omit difference density map (contoured at 2.4σ) as described for Figure 3. The same color scheme as Figure 3 is used with the addition that the glutamate tail of the inhibitor is red and broken lines represent potential hydrogen bonds. Phe113 is depicted as a semitransparent object. (b) A representation of the active site with the ligand in similar fashion to Figure 4.
The cofactor, when removed from the protein model, has an accessible surface area (ASA) of 915 Å². In the binary complex the ASA drops to about 110 Å², and a further decrease to 65 Å² accompanies binding of substrate/product. The relatively small pterin substrates or products have ASA values of about 410 Å² in isolation and around 50 Å² when bound in the active site formed by PTR1:NADP⁺. Almost 90% of the molecular surface is occluded by interactions head-on with the cofactor, sideways with Arg17 and Tyr181, and above and below with the nicotinamide and Phe113, respectively (Figures 4 and 5).

These crystal structures confirm that the substrates bind in a single orientation and, in conjunction with biochemical data, clearly define a sequential two-step reduction mechanism. The first catalytic step resembles that of other SDR family members and exploits three residues to (a) position the nicotinamide moiety of the cofactor NADPH for hydride transfer (Lys198), (b) acquire a proton from the solvent (Asp181), and (c) pass this on to the substrate (Tyr194). The second reduction step, which occurs on the opposite side of the pterin, is similar to that postulated for DHFR. Nicotinamide again provides a hydride ion and an activated water molecule supplies the proton.

The PTR1 catalytic center appears to be relatively rigid, with the correct alignment of functional groups to support catalysis. Seven residues (Arg17, Ser111, Phe113, Asp181, Tyr194, Lys198 and Arg287) are important for creating the active site, substrate binding, or implicated in catalysis (Figure 5). These residues and the cofactor are amongst the most ordered parts of the structure, as indicated by the lower than average temperature factors (or B-factors, where \(B = 8\sigma^2\langle\Delta^2U\rangle\), and \(\Delta^2U\) is the mean displacement of atoms along the normal to reflecting planes, data not shown).

**CB3717 binding to PTR1**

CB3717 is an N10-substituted conjugated pterin-like molecule similar to MTX, but with two significant differences, 2-amino-4-oxoquinazoline and prop-2-inyl (propargyl) replacing the 2,4-pyrimidinedione moiety. The presence of the 4-oxo group on CB3717 suggests that the quinazoline head group should adopt the pterin-like binding mode as opposed to the MTX binding mode and this has indeed proven to be the case (Figure 6).

The quinazoline ring is sandwiched between the cofactor nicotinamide and Phe113 and positioned such that all functional groups participate in hydrogen bonding interactions. These interactions are with the O2 hydroxyl group and one of the phosphate oxygen atoms of the cofactor together with side-chains of Arg17 and Ser111 (Figure 6). The N1 atom of CB3717 is 3.6 Å distant from Tyr194 OH, and this may represent a weak interaction. The propargyl moiety forms only a few van der Waals interactions with the side-chains of Phe113 and Leu188, in contrast to the extensive van der Waals interactions observed for this moiety when CB3717 is bound in the active site of TS. The benzyl group, again using van der Waals interactions, associates with Leu188, Leu226, Leu228 and His241. At the glutamate tail there is a single weak potential hydrogen bond accepted from Tyr283. Ethylene glycol is observed binding between the inhibitor, with which it makes van der Waals contacts, and a polar section of the active site formed by Asp181 and Arg287 at the same site noted in the binary cofactor complex and the MTX complex. This ethylene glycol replaces several of the highly conserved and ordered water molecules observed in the other structures (Figure 4). Replacement of N5 and N8 of the ligand, as present on the substrates, with carbon atoms ablates polar interactions with Tyr194 and a water molecule adjacent to the Arg17 guanidine and the backbone amide nitrogen atom at Val230.

The para-aminobenzoate (pABA)-glutamate tail of the inhibitor is poorly ordered, as reflected by the thermal parameters, which exceed 80 Å², and less well-defined electron density associated with this group. This feature is shared with MTX and is likely a consequence of the shape of the PTR1 ligand-binding cavity, which is relatively wide just above the catalytic center with a concomitant lack of specific interactions formed between the ligand and the enzyme. A biological consequence of this spacious entry into the active site is that PTR1 can process a wide range of pteridine compounds, including conjugated pteridines such as DHF.

MTX is a more potent inhibitor of DHFR (IC₅₀ 5 nM) than of PTR1 (IC₅₀ 1.1 μM), mainly because the pABA group makes extensive interactions, including two direct salt-bridge associations with basic residues in the DHFR active site. The lack of affinity of PTR1 for the pABA-Glu tail of MTX is demonstrated indirectly by the inhibitor TAQ, which is essentially just the pterin-component of MTX. TAQ adopts an identical orientation in the active site of PTR1 and binds with an IC₅₀ comparable to that of MTX.

In a similar fashion, CB3717 is a highly potent inhibitor of TS with IC₅₀ values in the range of 30–60 nM. When bound to *E. coli* TS, for example, the glutamate moiety binds directly to His51 and Ser54, and exploits numerous solvent-mediated hydrogen bonding links to the enzyme to form a stable complex (Protein Data Bank (PDB) code 1AN5). In addition, the propargyl and pABA groups participate in extensive van der Waals interactions with hydrophobic side-chains in TS.

The molecular conformation of CB3717 in the PTR1 ternary complex has an ASA of 200 Å², increasing to 765 Å² when the inhibitor is considered in isolation. When bound in the *E. coli* TS ternary complex the inhibitor in isolation, with a slightly different conformation, has an ASA of 710 Å², which decreases to only 90 Å² upon binding. The inhibitor is bound more tightly by
E. coli TS, assisted by large-scale adjustments of protein secondary structure to form a closed conformation about the drug. The increased ASA observed for the drug in the PTR1 complex is principally due to the tail of the molecule stretching out onto the surface of the ligand-binding site. The new structure and comparisons with the MTX and TAQ complexes suggests that the pABA-Glu moiety contributes little to binding PTR1 and could be dispensed with in the design of novel and potent inhibitors.

4-Oxo-2-amino quinazolines like CB3717 have poor affinity for PTR1, with IC50 values > 10 µM in all cases that have been examined. This affinity is similar to that of the pteridine substrates for PTR1. The loss of the polar interactions with N5 and N8 in the quinazolines cannot fully account for the poor affinity of PTR1 for CB3717, because several 6-substituted 2,4-diaminoquinazolines bind to the PTR1-NADPH complex with values of Kd! 20 nM. Replacement of the 4-oxo with 2-amino functionality seems to be a necessary but not sufficient feature for potent inhibition of PTR1 by quinazolines.

**Trimethoprim binding to PTR1**

TOP is a potent DHFR inhibitor and structural studies have revealed that it binds to the enzyme with the diaminopyrimidine moiety mimicking a pterin ring system with the TOP N4 replacing the pterin N8. The association of TOP with PTR1, with an IC50 of 12 µM, is very different. Here, the diaminopyrimidine is displaced by approximately 2.5 Å from the pterin-equivalent binding position (Figure 7). This can be explained by steric restrictions imposed on the trimethoxyphenyl tail of the inhibitor, in particular by Phe113 and Leu188. This prevents the diaminopyrimidine group from inserting deeply enough into the binding pocket to exploit the hydrogen bonding opportunities with the protein and cofactor, and to maximize van der Waals interactions with Phe113 and the nicotinate.

Only one well-defined direct hydrogen bond is formed between TOP and PTR1 via donation from N2 to the hydroxyl group of Tyr194. A water molecule is trapped between the diaminopyrimidine and cofactor participating in hydrogen bonds with TOP N2, a cofactor phosphate and Ser111. This water molecule occupies the position of N2 observed in the pterin complexes. TOP N1 is approximately equidistant (3.6 Å) from Asp181 OD1 and Tyr194 OH, and this may reflect the presence of a weak three-center hydrogen bond.

The 2,4-diaminopyrimidine ring of TOP carries four functional groups, two hydrogen bond donor (N2 and N4) and two acceptor groups (N1 and N3). When TOP binds to DHFR it makes use of all functional groups to form hydrogen bonds directly with the protein or mediated by a well-ordered water molecule. The pyridine N3 atom is worth further comment about an interaction, which so far has been overlooked. This group is 3.4 Å distant from Trp6 Cα placed to participate in a C–H$\cdots$N type of interaction similar to the C–H$\cdots$O type of interactions discussed previously. Although this is not a strong attractive force, it would reduce the significant destabilization likely to occur in the presence of an unfulfilled hydrogen bond acceptor.

There is rotational freedom about the C5–C7 and C7–C8 bonds in TOP (Figure 2), and conformational variability contributes to this molecule’s enhanced inhibition of bacterial DHFR, compared to the eukaryotic enzymes, as the drug adapts to binding in the active site. Also, in common with TS, conformational changes of DHFR (in particular of a loop at the edge of the catalytic center and the two sub-domains of the enzyme) accompany the binding of ligands in the active site. In contrast, the residues that form the substrate-binding site of PTR1 are well ordered and when cofactor is present, the amount of space available where a ligand can bind is highly restricted. This suggests that the limited conformational flexibility displayed by TOP...
would not improve binding to PTR1, due in particular to the side-chain positions of Leu188, Leu226, Leu229 and His241.

The molecular conformation of TOP in the PTR1 ternary complex has an average ASA of 80 Å² and 500 Å² when considered in isolation. In the *M. tuberculosis* DHFR ternary complex (PDB code 1DG5), the inhibitor in isolation adopts a slightly different conformation (not shown) but with a similar ASA of 500 Å², which decreases to 70 Å² upon binding. Although the ASA for TOP bound to PTR1 or DHFR is comparable, the loss of hydrogen bonding interactions together with reduced van der Waals associations contribute to the differences in inhibition against the two enzymes.

The inhibitory properties of 15 2,4-diaminopyrimidine derivatives against PTR1 have been assayed\(^1\) and the TOP complex now provides a clear explanation of the trends observed. Most 2,4-diaminopyrimidines with phenyl substituents at C5 and bulky substituents at C6 (Figure 2) are very poor inhibitors, with IC\(_{50}\) values ranging from 50 μM to in excess of 300 μM, most likely because of steric restrictions in the active site imposed by Phe113 and Leu188.

Two 2,4-diaminopyrimidines with IC\(_{50}\) values of ~3 μM have chlorophenyl substituents at C5 and ethyl groups at C6. Some of the molecules previously tested would, in addition to steric repulsion, suffer by virtue of positioning a hydrophobic group in a polar region of the active site near Asp181 and Arg287\(^9\). However, 2,4-diaminopyrimidines with 5-propylphenyl, or 5-butylphenyl substituents were improved inhibitors with IC\(_{50}\) values <1.5 μM, with the most potent of this series having a \(K_d\) value of ~30 nM for the PTR1-NADPH complex. Flexible C3 or C4 tethers attached to either C5 or C6 (Figure 2) might circumvent the steric restrictions imposed by the PTR1 active site and allow the 2,4-diaminopyrimidine to bind further in toward the cofactor, perhaps adopting the pterin-like binding mode. It would be of interest to investigate if adjustment of the substituent conformations to optimize van der Waals interactions with the enzyme could also occur. Such a mode of inhibition offers scope for future design of more potent inhibitors.

### Concluding Remarks and Implications for Inhibitor Design

The use of combinations of drugs, each with independent modes of action, has been shown to improve efficacy in some treatments without increasing toxicity, and with the additional and significant benefit of providing mutual protection against drug resistance. Of particular note with respect to parasitic infections are combinations of dapsone with chlorproguanil or pyrimethamine to combat malaria.\(^3\) For the treatment of bacterial infections, the combination of TOP with sulfonamide drugs is often used.\(^3\) With respect to the development of antifolate drugs to treat trypanosomatid infections, we note three valuable enzyme activities, DHFR-TS and PTR1. A determination of whether it is reasonable to expect that a single molecule can be obtained that is a potent inhibitor of two or more of these enzymes, or whether different compounds will be needed for the different enzymes, would indicate how to most productively pursue inhibitor discovery.

Gangjee *et al.* have shown that dual inhibition of DHFR and TS is feasible\(^40\) and, as discussed, MTX is an inhibitor of both DHFR and PTR1. Furthermore, a 2,4-diaminoquinazoline identified previously inhibited both PTR1 and DHFR with potency of ~30 nM, although that compound, like MTX, also inhibited human DHFR.\(^1\) On the basis of steric considerations and arrangement of functional groups, the TS, DHFR, and PTR1 active sites all have some similarities, yet each has distinctive features. In structures of ternary complexes of TS and PTR1, the substrates or inhibitors display extensive interactions with the enzyme and with the other ligand. For example, in PTR1 complexes there are often hydrogen bonds and strong π-stacking associations involving Phe113 and the nicotinamide cofactor. These structural features mandate the ordered binding seen both with TS and PTR1. DHFR has different structural features that allow it to bind its ligands in either order, albeit with a kinetic preference. DHFR and TS undergo extensive conformational changes upon ternary complex formation, whereas PTR1 is more rigid. DHFR and TS structures show strong associations with the pABA group of various ligands, whilst in PTR1 this group is bound only loosely. PTR1 offers steric restrictions preventing access, for some compounds, to the optimum pterin-like binding position. Both DHFR and PTR1 can each bind tightly the 2,4-diaminopteridine, 2,4-diaminoquinazolines, or 2,4-diaminopyrimidine scaffolds, though in a reverse orientation from that observed for the corresponding 4-oxo-2-amino molecules. TS, on the other hand, has no or very poor affinity for 2,4-diaminopteridine and 2,4-diaminoquinazolines.

There are already a significant number of inhibitors of DHFR-TS with well-characterized pharmacokinetics and impressive affinity for their targets. Since TS is one of the most highly conserved of enzymes, it might seem daunting to consider trying to inhibit a pathogen enzyme, given the deleterious affects likely to occur on human TS. Nevertheless, recent studies have shown conclusively that specificity towards microbial TS inhibition can be achieved,\(^32,33\) thereby providing encouragement for such an approach. Certainly, less toxic inhibitors of human TS might prove useful for antimicrobial drug development. However, at this stage we suggest that the priority should be development of PTR1 inhibitors likely to complement existing drugs that target DHFR, or that are capable of inhibiting both PTR1 and DHFR, with requisite selectivity against human DHFR.

Future work developing such PTR1 inhibitors can
be based on our enzyme–ligand complexes by extending from those sections of the molecules shown to be important for binding. The presence of a solvent-filled cavity, occupied by conserved water molecules (or ethylene glycol), and lined by hydrophilic residues (Asp181 and Arg287) suggests a suitable region to target by modification of the pterin, quinazoline or 2,4-diaminopteridine framework.

### Experimental

#### Materials

Bioterpin and THB were obtained from Schirks Laboratories (Jona, Switzerland) and other reagents were purchased from Sigma-Aldrich. CB3717 was provided by Professor A. Jackman.

#### Crystallization and data collection

Recombinant L. major PTR1 was purified and crystallized following published methods. Briefly, ligand solutions were prepared fresh (1 mM cofactor, 1 mM ligand in 20 mM dithiothreitol buffered with 20 mM sodium acetate (pH 5.3)) and mixed in tenfold excess with the enzyme solution in the same buffer. The enzyme–ligand mixtures were incubated on ice for an hour, and with the enzyme solution in the same buffer. The enzyme–sodium acetate (pH 5.3)) and mixed in tenfold excess ligand in 20 mM dithiothreitol buffered with 20 mM solutions were prepared fresh (1 mM cofactor, 1 mM

The asymmetric unit contains a tetramer (subunits are labeled A to D). The refinement protocol started with the PTR1:NADP⁺:DHB complex (PDB code 1E92), and applied rigid-body refinement (REFMAC) and simulated annealing (CNS) to that model. Cycles of model-map inspection (O), identification of solvent molecules and restrained least-squares refinement (initially with CNS then REFMAC) followed. Coordinates and topologies for ligands were obtained from HIC-Up. Throughout the refinement, non-crystallographic symmetry (NCS) restraints were applied to the four subunits in the asymmetric unit, excluding residues around several flexible loops and those that occupied clearly different conformations in some chains. Completely disordered residues have been omitted from the models, while partially ordered side-chains were built in a chemically sensible conformation and assigned low occupancies. Some density features have been interpreted as molecules of the cryo-protectant ethylene glycol. For the TOP complex, convincing electron density for the inhibitor was associated with two out of the four subunits in the asymmetric unit. Model geometries were analyzed with PROCHECK and WHAT_CHECK, and statistics are given in Table 1. Figures were produced using MOLSCRIPT, PyMOL, and Raster3D.

#### Protein Data Bank accession codes

Coordinates and structure factors have been deposited with the Protein Data Bank (with accession codes 2BF0, 2BF7, 2BFP, 2BFA, and 2BFM) and are available for immediate release upon acceptance.

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† http://pymol.sourceforge.net/


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